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SHORT COMMUNICATION

Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase by Digoxin Covalently Bound to Sepharose

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SUMMARY

A procedure for the synthesis of a series of digoxin-derivatized Sepharose compounds is described. These compounds inhibit a soluble $(Na^+ + K^+)$—ATPase from rat brain to a degree paralleling the length of the hydrocarbon bridge.

The covalent coupling of various ligands to insoluble polymers is a useful approach in the study of enzyme-substrate and drug-receptor interactions. Several enzyme systems have been successfully purified on columns of Sepharose to which specific enzyme inhibitors were covalently bound (1, 2). Soluble estrogen receptors from rat uterus were selectively removed from tissue homogenates by incubation with estrogen-derivatized resins (3). Insulin covalently coupled to Sepharose was shown to increase glucose utilization and to suppress hormone-stimulated lipolysis in isolated fat cells without entering the cell interior (4). Columns of Agarose, covalently linked to concanavalin A, have been shown specifically to retain mouse erythrocytes (5).

This laboratory has performed a series of studies on the locus of action of digoxin. Cultured beating heart cells respond to digoxin covalently linked to human serum albumin with positive chronotropic and inotropic responses, even though the complex does not enter the cell (6). We have further shown that this digoxin-albumin complex specifically inhibits an $(Na^+ + K^+)$-ATPase from rat heart and brain (6). To characterize this surface interaction further, we report here on the synthesis of a series of Sepharose-digoxin derivatives and their effect on a solubilized membrane $(Na^+ + K^+)$-activated, Mg^{2+}-dependent ATP phosphohydrolase (EC 3.6.1.3, hereafter called ATPase) from rat brain.

The preparation of ω-aminoalkyl derivatives of Sepharose was accomplished according to the method of Cuatrecasas (7). Sepharose 4B was activated with cyanogen bromide (300 mg/ml of packed Sepharose) and allowed to react with an aqueous solution (2 mmoles/ml of packed Sepharose) of ethylenediamine or 3,3′-diaminodipropylamine or with a solution (2 mmoles/ml of packed Sepharose) of decamethylenediamine in 50% dimethylformamide.

The reaction products were thoroughly washed with distilled H_2O and checked for reactive amino groups by the sodium 2,4,6-trinitrobenzenesulfonate color test (8). The alkylated Sepharose was washed until a concentrated sample of effluent wash (200 ml of effluent, lyophilized and brought to to 1.0 ml) gave a negative color test. The washed Sepharose beads were then sub-

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jected to the color test. A positive reaction of the washed beads was taken as the criterion of bound aminoalkyl groups. Addition of the diamine compound to beads that had not been treated with cyanogen bromide resulted in the absence of detectable bound amino groups.

The coupling of digoxin to the terminal amino group of the aminoalkyl-derivatized Sepharose was accomplished by a modification of the method of Smith and Butler (9). To a suspension of 800 mg of digoxin per 50 ml of packed, alkylated Sepharose in 40 ml of absolute ethanol were added 2×10^6 dpm of tritiated digoxin ([12α-^{3}H] digoxin; specific activity, 2 Ci/mmole). This suspension was subjected to periodate oxidation (0.05 M) until the solution became clear. The reaction was stopped with the addition of 1.2 ml of 1.0 M ethylene glycol, and the mixture was added to a suspension of alkylated Sepharose. The suspension was allowed to react at room temperature until the pH, which was maintained at 9.5 by the dropwise addition of a 5% solution of K_2CO_3, became stabilized at 9.0–9.5. The reaction mixture was then exposed to reduction by sodium borohydride (0.6 g), and the resultant product was exhaustively washed on a coarse filter in a Buchner funnel with 50% dimethylformamide.

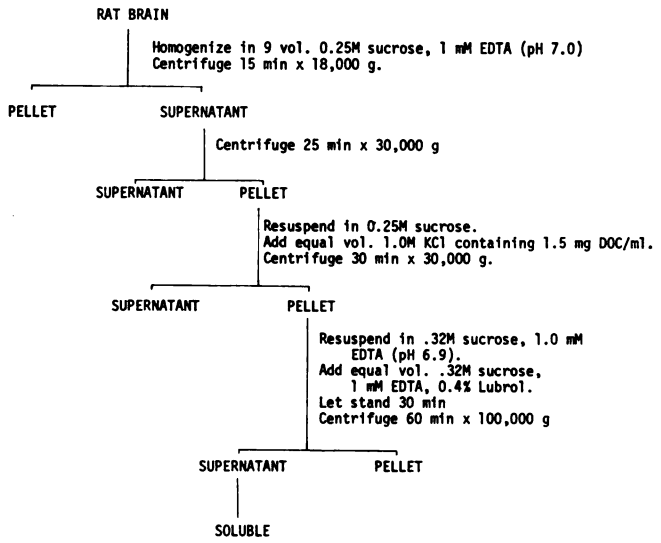
The beads were then packed in a column and washed with 50% dimethylformamide. After 1 week of column washing, a 200-ml sample of effluent was lyophilized, brought to 1.0 ml, and counted in a liquid scintillation counter to assay for unbound digoxin. A 1.0-ml fraction of the column-washed, digoxin-derivatized Sepharose was hydrolyzed with 0.1 N HCl at 80° for 1 hr and counted for bound digoxin. Absence of counts from the column effluent was taken as proof that the radioactivity present in the beads signified covalently bound digoxin. Absence of a positive trinitrobenzenesulfonate color test with the digoxin-derivatized beads was used as a criterion for the loss of reactive groups. Exposure of periodate-oxidized digoxin to non-aminoalkylated Sepharose resulted in the immediate washout of digoxin from the beads. Similarly, exposure of aminoalkylated Sepharose to non-oxidized digoxin resulted in the absence of radioactivity in the beads after washing.

$(Na^+ + K^+)$-ATPase was solubilized from rat brain microsomes with 0.2% Lubrol according to the procedure of Shirachi *et al.* (10). Microsomes were prepared as shown in Scheme 1, by a modification of the method of Schwartz *et al.* (11). The solubilized enzyme preparations were stable for at least 1 month at −20°. The Mg^{2+}-activated component constituted approximately 7% of the total activity assayed in the presence of Na^+, K^+, and Mg^{2+}. The concentrations of constituents used in the enzyme assays were as follows: TrisATP, 2.5 mM; NaCl, 100 mM; KCl, 10 mM; $MgCl_2$, 2.5 mM; and Tris-Cl, 100 mM (pH 7.4), in a total volume of 3 ml.

Approximately 50 μg of enzyme protein were added to each assay flask. Incubations were begun with addition of substrate and continued for 30 min at 37° in 25-ml Erlenmeyer flasks placed in a shaking water bath. The reaction velocity was linear over this time period and was directly proportional to enzyme concentration.

Liberation of inorganic phosphate was measured by a modification of the method of Fiske and SubbaRow (12). Reactions were stopped by the addition of cold trichloracetic acid to a final concentration of 5%. The resulting suspension was centrifuged for 10 min at 3000 × *g*. The pellet contained enzyme protein, Sepharose, and a filmy residue presumably related to the Lubrol present in the enzyme preparation. The supernatant fraction was clear. The phosphate assay was then carried out in the conventional manner.

Table 1 displays the quantity of digoxin bound to the Sepharose as a function of hydrocarbon bridge length. Increasing the chain length resulted in a higher yield of bound ligand. That this increase in digoxin binding was secondary to a more complete alkylation reaction was suggested by the following observations. The 2,4,6-trinitrobenzenesulfonate test indicated approximately a 5-fold increase in titratable amino groups for the decamethylene (C_{10}) alkyla-



SCHEME 1. *Procedure used to prepare solubilized ATPase from rat brain*

The procedure was modified from that of Schwartz *et al.* (11), and solubilization was performed according to Shirachi *et al.* (10).

TABLE 1

Amount of digoxin bound to Sepharose derivatives

See the text for experimental details.

Preparation	Digoxin
	μmole/ml Sepharose
Sepharose–C_2-digoxin[a]	0.16 ± 0.01
Sepharose–C_6-digoxin[b]	0.48 ± 0.015
Sepharose–C_{10}-digoxin[c]	0.67 ± 0.01

[a] C_2 = ethylenediamine.
[b] C_6 = 3,3′-diaminodipropylamine.
[c] C_{10} = decamethylenediamine.

tion over the ethylenediamine (C_2) alkylation. The number of 3,3′-diaminodipropylamine (C_6) titratable groups fell between the C_2 and C_{10} values. (Equimolar standard solutions of unbound C_2, C_6, and C_{10} diamino compounds reacted to the same degree with 2,4,6-trinitrobenzenesulfonate.) Thus the extent of the alkylation reaction may determine the amount of digoxin bound to the beads.

Table 2 displays the results of a typical incubation of enzyme and derivatized Sepharose. The final concentration of bound digoxin in the incubation flasks was 0.16 μM. The table shows that inhibition of the enzyme is a function of hydrocarbon bridge length, as expected. Increasing the length of the hydrocarbon bridge has been shown to improve the adsorption of several enzymes to various Sepharose-inhibitor derivatives (1, 7). The longer hydrocarbon bridge may decrease the steric hindrance generated by the Sepharose matrix, thus facilitating the inhibitor-enzyme interaction.

The aminoalkylated beads alone had no effect on the enzyme, demonstrating that inhibition was due to the digoxin ligand and not to the hydrocarbon bridge. These derivatized Sepharose compounds also inhibit a sodium iodide- and deoxycholate-treated particulate preparation of ATPase from rat heart to the same degree at similar concentrations (data not shown).

The applications of these Sepharose derivatives are numerous. We have attempted to employ them in our beating heart cell system *in vitro* (13) without success, as the beads themselves occlude the light path and prevent photoelectric recording of the heart cell contraction. These compounds might display activity in other heart preparations, however, such as the isolated papillary muscle or the open-chested dog. Such a demonstration would lend further support to a surface

TABLE 2

Inhibition of soluble ($Na^+ + K^+$)-ATPase by Sepharose derivatives

Inhibition is directly related to hydrocarbon bridge length and is specific for the digoxin ligand.

All preliminary incubations were conducted at 20°. Values are the averages of triplicate determinations.

Conditions	ATP hydrolyzed	Inhibition
	μmoles p_i/hr/mg protein	%
Minus ($Na^+ + K^+$)	3.6	
Complete ($Na^+ + K^+ + Mg^{2+}$)	49.1	
+ Sepharose-C_2-digoxin[a]	37.5	17.5
+ Sepharose-C_6-digoxin[b]	33.1	27
+ Sepharose-C_{10}-digoxin[c]	26.4	43
+ Sepharose-C_2[d]	45.0	1
+ Sepharose-C_6[d]	45.5	
+ Sepharose-C_{10}[d]	45.4	

[a] Digoxin, 0.16 μM (1.0 ml of packed derivatized Sepharose), was added 5 min before the beginning of the reaction.

[b] Digoxin, 0.16 μM (0.33 ml of packed derivatized Sepharose), was added 5 min before the beginning of the reaction.

[c] Digoxin, 0.16 μM (0.24 ml of packed derivatized Sepharose), was added 5 min before the beginning of the reaction.

[d] Addition of 1.0 ml 5 min before the beginning of the reaction.

action hypothesis for digoxin on the heart muscle cell.

Since the digoxin-derivatized Sepharose affords substantial inhibition of the enzyme at concentrations very near those employed in inhibition studies with free glycosides, our results raise the possibility of ATPase purification by affinity chromatography. The complete purification of ATPase has not yet been accomplished. Interpretation of numerous kinetic studies, all performed on enzyme preparations containing varying amounts of impurities, has therefore been difficult. Several investigators have reported on the solubilization of the enzyme with retention of its particulate kinetic characteristics, as well as its sensitivity to cardiac glycoside inhibition (14–16). It has been repeatedly demonstrated that a stable intermediate is formed between cardiac glycosides and ATPase (17–19). The degree of inhibition of ATPase activity is related to the degree of glycoside binding (20). Since the conditions under which the intermediate is formed markedly affect its stability (17), passage of a solubilized preparation of ATPase over a column prepared from digoxin-derivatized Sepharose might selectively retard ATPase-related protein under optimal binding conditions.

Our preliminary attempts to purify a solubilized ATPase preparation with Sepharose 4B–decamethylene-digoxin columns have been unsuccessful despite the use of various combinations of salt solutions for loading and elution. Although no ATPase activity has yet been recovered, several protein peaks have appeared in the eluate, suggesting that the enzyme might adhere very tightly to the column. As an alternative approach, we have synthesized a Sepharose 4B–decamethylene-dihydrodigoxin derivative (a less potent inhibitor of the enzyme) and are attempting to elute the enzyme from it with a salt solution containing a more potent competing inhibitor, such as ouabain or digoxin.

REFERENCES

1. P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S. A.* **61**, 636 (1968).
2. W. W.-C. Chan and M. Takahashi, *Biochem. Biophys. Res. Commun.* **37**, 272 (1969).
3. B. Vonderhaar and G. C. Mueller, *Biochim. Biophys. Acta* **176**, 626 (1969).
4. P. Cuatrecasas, *Proc. Nat. Acad. Sci. U. S. A.* **63**, 450 (1969).
5. G. M. Edelman, U. Rutishauser and C. F. Millette, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 2153 (1971).
6. T. B. Okarma, P. Tramell and S. M. Kalman, *J. Pharmacol. Exp. Ther.* In press.
7. P. Cuatrecasas, *J. Biol. Chem.* **245**, 3059 (1970).
8. J. K. Inman and H. M. Dintzis, *Biochemistry* **8**, 4074 (1969).
9. T. W. Smith, V. P. Butler and E. Haber, *Biochemistry* **9**, 331 (1970).
10. D. Y. Shirachi, A. A. Allard and A. J. Trevor, *Biochem. Pharmacol.* **19**, 2893 (1970).
11. A. Schwartz, H. S. Bachelard and H. McIlwain, *Biochem. J.* **84**, 626 (1967).

12. C. H. Fiske and Y. J. SubbaRow, *J. Biol. Chem.* **66**, 375 (1925).
13. T. B. Okarma and S. M. Kalman, *Exp. Cell Res.* **69**, 128 (1971).
14. F. Medzihradsky, M. H. Kline and L. E. Hokin, *Arch. Biochem. Biophys.* **121**, 311 (1967).
15. A. Kahlenberg, N. C. Dulak, J. F. Dixon, P. R. Galsworthy and L. E. Hokin, *Arch. Biochem. Biophys.* **131**, 253 (1969).
16. M. H. Kline, T. D. Hexum, J. L. Dahl and L. E. Hokin, *Arch. Biochem. Biophys.* **147**, 781 (1971).
17. J. C. Allen, R. A. Harris and A. Schwartz, *Biochem. Biophys. Res. Commun.* **42**, 366 (1971).
18. T. Akera and T. M. Brody, *J. Pharmacol. Exp. Ther.* **176**, 545 (1971).
19. A. Yoda and L. E. Hokin, *Biochem. Biophys. Res. Commun.* **40**, 880 (1970).
20. A. Schwartz, J. C. Allen and S. Harigaya, *J. Pharmacol. Exp. Ther.* **168**, 31 (1969).